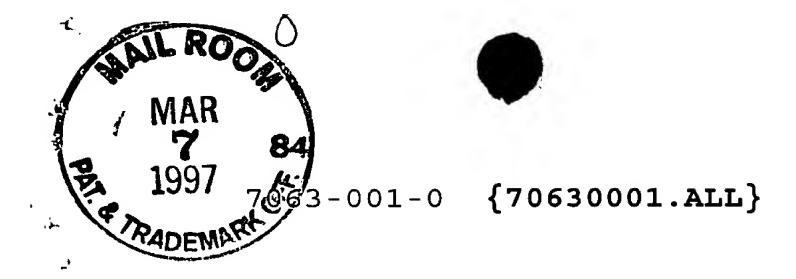
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TITLE OF THE INVENTION

STRAINS OF BACTERIA AND PHARMACEUTICAL COMPOSITION

CONTAINING ONE OR MORE OF SUCH STRAINS AND USE OF SAME FOR

PREVENTING AND TREATING DISEASES ASSOCIATED WITH OR CAUSED

BY ALTERED METABOLISM OF BILE ACIDS

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention relates to strains of bacteria and pharmaceutical compositions containing one or more of such strains and the use of same for preventing and treating diseases associated with or caused by an altered metabolism of bile acids.

<u>Discussion of the Background</u>:

Hepatic bile is a pigmented isotonic fluid with an electrolyte composition resembling blood plasma. Major components of bile include water (82 percent), bile acids (12 percent), lecithin and other phospholipids (4 percent), and unesterified cholesterol (0.7 percent). Other constituents include conjugated bilirubin, proteins, electrolytes, mucus and the final products of hepatic

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transformation of drugs, hormones, etc. The liver production of bile, in basal conditions, is approximately 500-1000 ml/day.

The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the liver, conjugated with glycine or taurine, and excreted into the bile. Secondary bile acids, including deoxycholic acid (DCA) and lithocholic acid (LA), are formed in the colon as bacterial metabolites of the primary bile acids. Other bile acids, called tertiary bile acids (e.g.: ursodeoxycholic acid - UDCA), are formed in the gut following the enzymatic epimerization of -OH groups on sterol rings by the intestinal flora.

In normal bile, the ratio of glycine to taurine conjugates is about 2:1, while in patients with cholestasis, increased concentrations of sulfate and glucuronide conjugate of bile acids are often found. The intestinal microflora transforms the bile acids into different metabolites. These biotransformations include the hydrolysis of the bond between the bile acid and taurine or glycine, with formation of unconjugated or free bile acids and taurine or glycine. The unconjugated bile

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acids are therefore made available for the oxidation of the hydroxylic groups in positions C3, C7, and C12 and for the dehydroxylation in positions 7α and 7β . This latter transformation leads to the formation of the secondary bile acids DCA and LA. The primary bile acids, deconjugated bile not transformed, and the secondary biliary acids are reabsorbed from the gut lumen and enter the portal bloodstream, then are taken up by hepatocytes, conjugated with glycine or taurine and resecreted into the bile (enterohepatic circulation).

Normally, the bile acid pool circulates approximately 5 to 10 times daily. Intestinal absorption of the pool is about 95% efficient, so fecal loss of bile acids is in the range of 0.3 to 0.6 g/day. The fecal loss is compensated by an equal daily synthesis.

For this reason the composition of the pool of biliary acids present in the bile is the result of complex interactions occurring between the liver and the microflora enzymes.

Deconjugation activity is a characteristic shared by many bacteria, aerobes and anaerobes, but is particularly common among the obligate anaerobic bacteria, i.e.

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Bacteroides, Eubacteria, Clostridia, Bifidobacteria, etc.

The majority of the bacteria is active against both glycine and taurine conjugates; however, some of them have a certain degree of specificity, depending on the bound amino acid and the number of hydroxides bound to the steroid nucleus. The free biliary acids obtained following the action of the bacterial hydrolases can undergo the oxidation of the hydroxide groups present at the C3, C7, and C12 positions by the hydroxysteroidodehydrogenase.

The interest in the metabolic disorders of biliary acids comes from the hypothesis that biliary acids and/or metabolites thereof are involved in the pathogenesis of some hepato-biliary and gastroenterologic diseases: biliary dyspepsia, cholelithiasis, acute and chronic hepatopathies, inflammatory diseases of the colon, etc.

Very often in literature the hydrophobicity of the bile acid is correlated with detergency; the secondary bile acids are more hydrophobic than the primary bile acids, the deoxycholic acid (DCA) being actually more detergent than the cholic acid (CA). Therefore an increased concentration of DCA in the bile may involve: a) an augmentation of the

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secretion of cholesterol, with increased saturation index;
b) a cytotoxic effect on the liver cells.

For this reason a qualitative modification of the bile acids pattern could be a decisive factor, especially in treating the above-mentioned pathologies.

Thus, there remains a need for effective bacterial strains or compositions that, by reducing the 7α -dehydroxylase activity and at the same time deconjugation, can be used for treating and/or preventing diseases associated with metabolic disorders of the biliary acids.

No bacteria strains have been found that are capable of qualitatively modifying the bile acid pattern in such a way.

SUMMARY OF THE INVENTION

Accordingly, it is one object of the present invention to provide novel strains of bacteria, in particular grampositive bacteria, which are useful for treating and/or preventing diseases associated with or caused by a metabolic disorder of biliary acids.

It is another object of the present invention to provide pharmaceutical compositions which contain one or



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more strains of such bacteria and are useful for treating and/or preventing diseases associated with or caused by a metabolic disorder of biliary acids.

It is another object of the present invention to provide a novel method for treating and/or preventing diseases associated with or caused by a metabolic disorder of biliary acids.

The foregoing and other objects, which will become more apparent during the following detailed description, have been achieved by the inventors, who have found bacteria strains having a reduced or zero 7α -dehydroxylase activity and a reduced or zero ability to deconjugate bile acids. This is in contrast with the previous known art. Accordingly, the present invention provides the use of such strains to modify the bile acid metabolism in a useful manner to prevent or treat diseases caused by or associated with metabolic disorders of biliary acids.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Thus, in a first embodiment, the present invention provides novel strains of bacteria which have a 7α -dehydroxylase activity of less than 50%, preferably less

than 25%, and a conjugated bile acid deconjugation activity of less than 50%, preferably less than 25%.

The essential features of the strains according to the present invention are defined in claim 1; specific strains having said features are defined in the dependent claims 2 to 11.

The present invention also provides a pharmaceutical composition for treating and/or preventing diseases associated with or caused by an altered metabolism of biliary acids, said composition comprising at least one bacteria strain according to the present invention. The essential features of the composition according to the present invention are defined in claim 12; specific embodiments of said composition are defined in the dependent claims 13 to 25.

In another embodiment, the present invention provides a method for treating and/or preventing diseases caused by or associated with an altered metabolism of biliary acids by administering to a patient in need thereof one or more strains of bacteria which have a 7α -dehydroxylase activity of less than 50%, preferably less than 25%, and a conjugated bile acid deconjugation activity of less than

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50%, preferably less than 25%, or a pharmaceutical composition containing one or more such strains of bacteria.

The essential features of said method are defined in claim 26; specific embodiments are defined in the dependent claims 27 to 36.

In the context of the present invention, the diseases associated with or caused by a metabolic disorder of the biliary acids include liver diseases and diseases of the digestive apparatus, such as blind loop syndrome, biliary gallstones, cirrhosis, chronic hepatopathies, acute hepatopathies, cystic fibrosis, intrahepatic cholestasis, intestinal inflammatory diseases, colonpathies, malabsorption. The present pharmaceutical compositions may also be used to prevent the onset of biliary gallstones in women during pregnancy or subsequent periods and in subjects undergoing weight-loss programs or diets.

The 7α -dehydroxylase activity of the bacteria strain should be less then 50%, preferably less than 25%. The 7α -dehydroxylase activity values are those measured by the method described in Example 1 below. Specifically, the 10^7 cells of the strain in question are incubated at 37° C for

48 hours, in 15 ml of the specific culture medium with the addition of 2 mg/ml of glycocholic acid (GCA) or 2 mg/ml of taurocholic acid (TCA), and then the amount of $7\alpha^{\chi\nu\beta}$ dehydroxylated product is measured. The percentage value for the 7α -dehydroxylase activity is calculated by the following formula:

 $7\alpha\text{-dehydroxylase} = \frac{\text{mass of }7\alpha\text{-dehydroxylated product}}{\text{activity}} = \frac{\text{after 48 hours of incubation}}{\text{mass of GCA or TCA at the}} \times 100$

+7,0100

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The $7\alpha\text{-dehydroxylase}$ activity for any given strain is the higher of the numbers measured for GCA and TCA.

Based on the above, the bacteria strain to be administered should in addition have a conjugated bile acid deconjugation activity of less than 50%, preferably less than 25%. The ability to deconjugate bile acid is determined by using the same incubation procedure described for measuring the 7α -dehydroxylase activity followed by measuring the amount of deconjugated product formed. The deconjugation activity is calculated using the following formula:

Deconjugation activity = $\frac{\text{mass of deconjugated GCA or TCA}}{\text{mass of GCA or TCA at the}} \times 100$

+1,0110

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The deconjugation activity for any given strain is the higher of the numbers measured for GCA and TCA.

The bacteria strains of the present invention may be administered enterically. Preferably, the bacteria strains of the present invention are administered orally.

Although a single bacteria strain may be administered, it is also possible to administer a mixture of two or more bacteria according to the present invention.

Although the exact dosage of bacteria to be administered will vary with the condition and size of the patient, the exact disease being treated, and the identity of the strains being administered, good results have been achieved by administering 10³ to 10¹³ cells of the bacteria/g, preferably 10⁸ to 10¹² of the bacteria strain/g. To achieve the good effects of the present invention, it is preferred that the strain be administered in an amount and a concentration sufficient to result in the intestines of the patient being populated with an important amount

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thereof. Thus, it is preferred that the strain be administered in a composition which contains 10³ to 10¹³ cells of the strain/g, preferably 10⁸ to 10¹² cells of the strain/g and that the composition be administered in such a regimen so that the patient receives 100 mg to 100 g of the strain/day, preferably 1 g to 20 g of the strain/day, for a period of 1 to 365 days, preferably 3 to 60 days in case of therapy, or according to periodical cycles in case of prophylaxis. The bacteria strain may be administered in any form suitable for enteral administration, such as capsules, tablets, or liquids for oral administration or liquids for enteral administration.

Typically, the administration of the bacteria strain according to the present invention can be prescribed after the diagnosis of metabolic disorders of the biliary acids. However, in the case of the prophylaxis of biliary gallstones, the strain may be administered when the subject is determined to belong to an at-risk population, such as becoming pregnant or beginning a weight-loss program or diet. In addition, the present strain of bacteria may be administered after a patient has had their gallbladder removed.

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In a preferred embodiment, the coadministration of lactulose is provided when the disease being treated is cirrhosis. Suitably, the lactulose is administered in an amount of 100 mg to 100 g/day, preferably 1 g to 20 g/day.

In another preferred embodiment, the coadministration of bile acid-based preparations, such as ursodeoxycholic acid or tauroursodeoxycholic acid, is provided. Suitably, the ursodeoxycholic or tauroursodeoxycholic acid is administered in an amount of 10 to 3,000 mg/day, preferably to 800 mg/day.

The present invention finally provides novel pharmaceutical compositions for treating and/or preventing the metabolic disorders of the biliary acids which comprise (a) one or more strains of bacteria having a 7α-dehydroxylase activity of less than 50%, preferably less than 25%, and a bile acid deconjugation activity of less than 50%, preferably less than 50%, preferably less than 25%, and (b) a pharmaceutically acceptable carrier. Preferably, the present pharmaceutical compositions contain the strain(s) of bacteria in a concentration of 10³ to 10¹³ cells/g, preferably 10° to 10¹² cells/g. The pharmaceutically acceptable carrier may be any which is suitable for enteral

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administration and is compatible with the strain of bacteria, such as dextrose, calcium carbonate together with different additional substances such as starch, gelatin, vitamins, antioxidants, stains or taste-improving substances.

As an optional component, the compositions of the invention may possibly contain a drug compatible with the bacteria employed and capable of potentiating the activity of the active ingredients present. Anticholinergic drugs, antihistamines, adrenergic, antiulcer, antiacid, antidiarroic, and anti-inflammatory drugs, sedatives, antipyretis, choleretics antirheumatic agents, analgesic drugs, diuretics, antiseptic agents, antilipemic hepatoprotective drugs and drugs active on the gastrointestinal motility (e.g., trimebutine) may be herein mentioned.

When treating cirrhosis, it is preferred that the pharmaceutical composition further comprise lactulose. Suitably, the composition will contain sufficient lactulose to result in the administration of 100 mg to 100 g/day, preferably 1 g to 20 g/day of lactulose. When treating biliary cirrhosis and chronic hepatitis, it is preferred

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that the pharmaceutical composition comprise bile acid-based preparations, such as ursodeoxycholic acid or tauroursodeoxycholic acid. Suitably, the composition will contain sufficient bile acid preparation to result in the administration of 10 to 3,000 mg/day of such bile acid preparations, preferably 50 to 800 mg/day of ursodeoxycholic acid or tauroursodeoxycholic acid.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1.

Strains of the following species have been tested:

Streptococcus thermophilus, Streptococcus faecium,

Lactobacillus acidophilus, Lactobacillus bulgaricus,

Lactobacillus plantarum, Bifidobacterium infantis. Each

strain (107 CFU) was cultivated in duplicate in specific

nutrient broths (15 ml); "CFU" means "colony forming

units".

List of the employed culture media depending on the

Bifidobacterium infantis:

different species

MRS + 0.5% glucose (added

after sterilization by

5 +T,0160

diluting a 20% sterile

solution)

Streptococcus thermophilus:

M17

All the remaining strains

MRS

Composition of the MRS broth:

10	g/liter	universal peptone	10.0g
	· ·	meat extract	5.0g
+T	,0161	yeast extract	5.0g
		D(+)-glucosé	20.0g
		potassium hydrogen phosphate	2.0g
15		Tween 80	1.0g
		dibasic ammonium citrate	2.0g
		sodium acetate	5.0g
		magnesium sulfate	0.1g
• •		manganous sulfate	0.05g

Preparation: dissolve 50 g/l in distilled water, sterilized at 121°C for 15 minutes - pH 6.5 ± 0.1 at 25°C Composition M17 broth (Merck):

HT0170		
+T,0/70 g/liter	soybean flour peptone	5.0g
	meat peptone	2.5g
	casein peptone	2.5g
	yeast extract	2.5g
5	meat extract	5.0g
	D(+)-lactose	5.0g
	ascorbic acid	0.5g
	sodium-β-glycerophosphate	19.0g
	magnesium phosphate	0.25g

10 Preparation: dissolve 42.5 g/l in distilled water, sterilized at 121°C for 15 minutes - pH 7.2 \pm 0.1 at 25°

Bifidobacterium infantis was cultivated under anaerobic conditions since it is known that it is an anaerobic bacterium. After 24 hours of incubation at 37°C to each tube was added an amount of bile salt equivalent to 30 mg in order to obtain a final concentration of 2 mg/ml. The bile acids employed are: glycocholic acid (GCA) and taurocholic acid (TCA), obtained from Sigma Chemicals. Each bile acid was added separately to each series of bacterial cultures.

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After 48 hours of incubation, isopropanol, 3 ml, was added for 2 minutes. Then it was centrifuged at 400 rpm for 15 minutes and the supernatant was collected (5 ml). The supernatant was kept refrigerated at -30°C until it was The percentage of conjugated bile salt present analyzed. was determined by HPLC (high performance liquid chromatography) utilizing a Gilson apparatus equipped with a detector Diode array mod 1000 and a Spherisorb 5 $\mu \mathrm{m}$ ODS 2 C18 reverse phase column, a mobile phase composed by methanol/buffered phosphate (20 mMol), pH 2.5 in water/acetonitrile/water (150:60:20:20 by volume), a fluid speed of 0.85 ml/min, at a wavelength of 205 nm; 100 μ l of the sample to be tested, dried under nitrogen, were extracted with 100 μl of the mobile phase containing as an internal standard 7α -OH-12 α -OH-dihydroxy-58-cholanic acid (Calbiochem U.S.A.) at a concentration of 2 mg/ml.

The recovery percentage of the bile acid incubated with the bacterial cultures was calculated by the ratio of the area of the bile acid to be detected (GCA or TCA) to the area of the internal standard. When the quantity of the conjugated bile acid found in the bacterial cultures after 48 hours of incubation was less than 50%, thin layer

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chromatography (TLC) was performed on silica 60 gel plates to detect the presence of CA and DCA, using a mobile phase of cyclohexane/isopropanol/acetic acid (30:10:1 by volume). On every plate, 20 μ l of the alcoholic extract of the sample, 20 μ l of a solution of CA and DCA, and 20 μ l of CA, 20 μ l of DCA, were spotted. The plates after development at room temperature, were treated with sulfuric acid and warmed at 145°C until the appearance of the colored spots.

The results of the deconjugation experiments (Table I) show that 5 out of the 16 strains tested with GCA were able to completely deconjugate the bile acid added to the culture, as previously reported in the literature and widely known to all researchers. Surprisingly, ten strains were able to deconjugate GCA but not completely, ranging from 9 to 90 percent (Table I). There was no difference among aerobic and anaerobic bacteria. Two strains, Streptococcus thermophilus YS 52 and Bifidobacterium infantis Bi 6 do not have any deconjugating activity for GCA. The strain YS 52 in addition does not attack the bile acid - taurine bond.

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Only one out of the 16 strains tested was able to totally deconjugate the TCA: the Bifidobacterium infantis Bi 6.

The results of the dehydroxylation experiments (Table II) show that only one (Bi 4) out of the 16 strains is able to completely dehydroxylate GCA. Six strains did not dehydroxylate at all: YS 52; SF 2; SF 4; LA 3; LA 10; and Bi 6. The other strains were able to dehydroxylate GCA but not completely, ranging from 9% to 90%. As to TCA, seven strains do not dehydroxylate it at all: YS 52; SF 3; LA 3; LA 10; LB 1; LB 7; and LB 77. One strain, Bi 6, dehydroxylated TCA completely; the other strains dehydroxylated TCA according to varying percentages.

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Table I

Percentage of deconjugation of GCA and TCA by bacterial cultures after 48 hours of incubation

BACTERIUM	ACCESSION NO.	GCA%	TCA%
Streptococcus thermophilus YS 46	I-1668	9	9
Streptococcus thermophilus YS 48	I-1669	17	11
Streptococcus thermophilus YS 52	I-1670	0	0
Streptococcus faecium SF 2		100	3
Streptococcus faecium SF 3	I-1671	27	0
Streptococcus faecium SF 4		100	12
Lactobacillus acidophilus LA 3		100	80
Lactobacillus acidophilus LA 10		100	95
Lactobacillus bulgaricus LB 1	I-1664	9	9
Lactobacillus bulgaricus LB 3	I-1665	20	12
Lactobacillus bulgaricus LB 7	I-1669	14	0
Lactobacillus bulgaricus LB 77	I-1667	80	0
Bifidobacterium infantis Bi 2		80	15
Bifidobacterium infantis Bi 3		90	10
Bifidobacterium infantis Bi 4		100	26
Bifidobacterium infantis Bi 6		0	100

+7,0210

+T,0720

Table II

Percentage of dehydroxylation of GCA and TCA by bacterial cultures after 48 hours of incubation

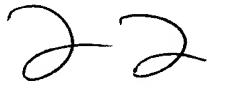
BACTERIUM	ACCESSION NO.	GCA%	TCA%
Streptococcus thermophilus YS 46	I-1668	•	9
Streptococcus thermophilus YS 48	I-1669	17	11
Streptococcus thermophilus YS 52	I-1670	0	0
Streptococcus faecium SF 2		0	3
Streptococcus faecium SF 3	I-1671	27	0
Streptococcus faecium SF 4		0	12
Lactobacillus acidophilus LA 3		0	0
Lactobacillus acidophilus LA 10		0	0
Lactobacillus bulgaricus LB 1	I-1664	•	0
Lactobacillus bulgaricus LB 3	I-166 \$	20	12
Lactobacillus bulgaricus LB 7	I-1666	14	0
Lactobacillus bulgaricus LB 77	I-1667	20	0
Bifidobacterium infantis Bi 2		80	15
Bifidobacterium infantis Bi 3		90	10
Bifidobacterium infantis Bi 4		100	26
Bifidobacterium infantis Bi 6		0	100

These strains have been deposited with the CNCM,

Collection Nationale de Cultures de Microorganismes,

Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cédex 15,

France, under the following accession numbers:



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	Streptococcus	thermophilus YS	3 46:	I-1668
	Streptococcus	thermophilus YS	8 48:	I-1669
	Streptococcus	thermophilus YS	5 52:	I-1670
•	Streptococcus	faecium SF 3:		I-1671
5	Lactobacillus	bulgaricus LB 1	. :	I-1664
	Lactobacillus	bulgaricus LB 3	:	I-1665
	Lactobacillus	bulgaricus LB 7	' :	I-1666
	Lactobacillus	bulgaricus LB 7	77:	I-1667

The following strains are on the contrary kept at the

10 Centro Ricerche Sitia-Yomo S.p.A., -strada per mercino 3
ZELO BUON PERSICO (MILAN) - ITALY, distinguished by the

below-reported identifiers:

SF 2

	Streptococcus faecium SF 4:	SF 4
15	Lactobacillus acidophilus LA 3:	LA 3
	Lactobacillus acidophilus LA 10:	LA 10
	Bifidobacterium infantis Bi 2:	Bi 2
	Bifidobacterium infantis Bi 3:	Bi 3
	Bifidobacterium infantis Bi 4:	Bi 4
20	Bifidobacterium infantis Bi 6:	Bi 6

Streptococcus faecium SF 2:

These results demonstrate that the majority of the strains tested by us have a low capability to deconjugate

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the bile acids and that there are strains that do not deconjugate at all. This observation is surprising in that it has not been known that the lactic acid bacteria deconjugated the biliary salts. Furthermore, it is evident that the enzymes of the strains are selective for the specific bile acid bound on the side chain. In this study, the clearest example is offered by the Bifidobacterium infantis Bi 6. This strain is not able to deconjugate the glycine-conjugated bile acid but is able to totally deconjugate the taurine-conjugated bile acid. Some other strains (LB 1, LB 7, LB 77, and SF 3) are unable to deconjugate TCA but are able to deconjugate GCA to a certain extent.

To conclude, strains have been discovered that have a weak or zero capability to deconjugate and dehydroxylate.

Example 2.

Three healthy volunteers were tested for their content of bile acids following treatment with a lactobacilli preparation containing 1x10¹¹ cells of *Streptococcus* thermophilus YS 52 per gram for a daily total of 6 g for 28 days. Before beginning the treatment and after 12 hours

starvation, the subjects were intubated and the gallbladder bile, following stimulation with ceruletide, was collected and frozen at -80°C. The gallbladder contraction was assessed by echography and the position of the tube, in the second portion of the duodenum was checked by Rx (fluoroscopy).

After a 4 week treatment, the subjects underwent a second intubation and collection of bile. The bile samples were then tested for their content of some bile acids as previously described. The results are shown in Table III.

+70250

Table III

	Patie	Patient #1		Patient #2		Patient #3	
Bile Acid	Before	After	Before	After	Before	After	
Glychenodeoxycholic	32	15	22	15	25	12	
Glycodeoxycholic	6	5	9	2	4	3	
Glycoursodeoxycholic	1	5	1	7	4	4	
Taurocholic	6	26	15	25	12	21	
Taurodeoxycholic	1	3	5	8	3	9	

NOTE: (the bile acids are listed following the hydrophilic capacity order, that is in inverse relation to detergency)

20 Taurocholic

Taurodeoxycholic

Glycoursodeoxycholic

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Glycodeoxycholic

Glychenodeoxycholic

This experiment is a confirmation of what is shown in Example No. 1, that is: a lower deconjugation in one of the primary bile acids if bacteria being the object of the present invention are administered. The achieved result is a longer maintenance of the primary bile acids in the enterohepatic circulation.

The properties of the bile acids are reported in the note to Table III. Thus, according to these results the administration of selected strains of bacteria can reduce the detergency property and therefore the cytolytic activity of the bile acids.

Example 3.

Fourteen patients with chronic hepatitis were treated with a bacterial preparation containing Streptococcus thermophilus YS 46 and YS 48 (two strains), and Lactobacillus bulgaricus LB 1, LB 7, and LB 77 (three strains). Each strain had been brought to a concentration of 150x10° cells per gram before being mixed with the others, to prepare a mixture containing the same parts by

weight of each strain. 6 grams per day of said mixture were administered for 28 days. Liver enzymes were measured before and after the treatment, and the results are shown in Table IV.

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Table IV

Influence of the Treatment with the Bacterial Mixture on Liver Enzymes Aspartate Transaminase (AST; SGOT) and alanine transaminase (ALT; SGPT)

	AST (SGOT) ALT (SGPT)	
Patient	Before	After	Before	After
#1	92	59	102	46
#2	89	67	96	42
#3	174	86	97	39
#4	121	91	102	66
#5	116	81	118	55
#6	156	87	94	76
#7	163	86	69	37
#8	78	64	122	57
#9	109	39	87	86
#10	166	70	102	48
#11	8 6	8 4	118	62
#12	131	83	96	79
#13	137	86	94	74
#14	84	87	144	114
Mean	119	71	102	63
Standard deviation	36	19	17	21
Significance Student t test for paired data	p<0.001 p<0.001		.001	

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Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.